

Application of a *Mycoplasma* Group-Specific PCR for Monitoring Decontamination of *Mycoplasma*-Infected *Chlamydia* sp. Strains

J. M. OSSEWAARDE,^{1*} A. DE VRIES,¹ T. BESTEBROER,¹ AND A. F. ANGULO²

Research Laboratory for Infectious Diseases¹ and Laboratory of Mycoplasma Research,² National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands

Received 28 June 1995/Accepted 1 November 1995

Mycoplasma contamination of biological materials remains a major problem. Most contaminations are caused by the use of *Mycoplasma*-contaminated cell lines. We adapted a *Mycoplasma* group-specific PCR to detect *Mycoplasma* contamination in cell lines and demonstrate its use in monitoring decontamination procedures with *Mycoplasma*-contaminated suspensions of *Chlamydia* spp. Three different methods were investigated: the use of *Mycoplasma*-specific antiserum in cell culture, physical separation by the combined use of enzymatic treatment and differential centrifugation, and the use of detergents. With these methods only incubation with Triton X-100 resulted in decontamination of *Mycoplasma*-contaminated suspensions of several laboratory strains of *Chlamydia pneumoniae*, *C. pecorum*, and *C. trachomatis*. Only one *C. pneumoniae* strain, UZG-1, was sensitive to Triton X-100 treatment. Since 39 of 40 throat swabs from patients with symptoms of an upper respiratory tract infection had positive reactions in the *Mycoplasma* group-specific PCR, this procedure could also have clinical significance in attempts to propagate *C. pneumoniae* strains from clinical specimens.

Contamination of biological materials, such as cell lines, viruses, and bacteria, with *Mycoplasma* spp. is a major problem. Although the class *Mollicutes* consists of >120 species, only 5 *Mycoplasma* species account for >95% of contaminations: two of bovine origin, *M. arginini* and *Acholeplasma laidlawii*; two of human origin, *M. orale* and *M. fermentans*; and one of porcine origin, *M. hyorhinis*. Although contamination of biological materials may originate from handling by humans or the use of animal sera, the main source is *Mycoplasma*-contaminated cell lines. When contaminated cultures are used, virtually every property of the cell line may be altered. As a result, properties and measured parameters of viruses and intracellular bacteria propagated in *Mycoplasma*-contaminated cell lines may also be altered. Especially when sensitive molecular methods are used to analyze the nucleic acid strains, the presence of small amounts of *Mycoplasma* DNA may significantly influence the results. Since *Mycoplasma* contamination is usually not visible on microscopic inspection of cell lines, periodic screening is highly recommended. Many detection methods have been reported, but recently developed PCR assays are probably the most sensitive (14).

Recently, we detected contamination of our cell lines by *M. arginini*. Although further spread of the contamination could be easily prevented by using a *Mycoplasma*-free cell line stored in liquid nitrogen, several propagated *Chlamydia* strains needed to be decontaminated. *Mycoplasma* contamination of *Chlamydia* strains has also been observed by other research groups (8). Mycoplasmas are relatively resistant to decontamination procedures. There is vast literature on different methods. Most of the methods were developed for decontamination of cell lines and are not suitable for decontamination of *Chla-*

mydia strains. Most antibiotics effective against *Mycoplasma* spp. are also inhibitory to *Chlamydia* spp. Several methods based on physical separation of the contaminating *Mycoplasma* spp. and the cells fail because *Chlamydia* spp., especially *Chlamydia pneumoniae*, has to be propagated in cell cultures assisted by centrifugation. Although members of the family *Rickettsiaceae* could be decontaminated by passage in animals (5), passage of hybridoma cells in mice is not always successful in removing *Mycoplasma* spp. (2, 13). Mycoplasmas have no cell walls, and their outer membranes are sensitive to organic solvents. Thus, suspensions of lipid-free viruses can easily be decontaminated by treatment with ether or chloroform (6). Membranes can also be disrupted by detergents. We used a specific antiserum against *M. arginini* (11), treatment with trypsin (10) and DNase, and treatment with detergents to decontaminate *Chlamydia* strains. The process of decontamination was monitored by a *Mycoplasma* group-specific PCR (18) and verified by culture.

MATERIALS AND METHODS

Chlamydia strains. *C. trachomatis* serovars D (strain IC-CAL-8) and L2 (strain 434-B) were a gift from S. Darougar, Institute of Ophthalmology, University of London, London, United Kingdom. *C. pneumoniae* TW-183 was purchased from the Washington Research Foundation, Seattle; strains CM-1, 2023, and 2043 were purchased from the American Type Culture Collection, Rockville, Md.; strains CWL-011, CWL-029, and CWL-050 were a gift from C. M. Black, Centers for Disease Control and Prevention, Atlanta, Ga.; strain GRO-21 was a gift from S. Farholt, Statens Seruminstitut, Copenhagen, Denmark; strain IOL-207 was a gift from J. Trehanar, Institute of Ophthalmology, University of London; and strain UZG-1 was a gift from M. van den Abeele, University Hospital, Ghent, Belgium. *C. pecorum* E58 was purchased from the American Type Culture Collection.

Patient specimens. Throat swabs from 40 patients consulting a general practitioner for symptoms of an upper respiratory tract infection were collected in 5.0 ml of gelatin-lactalbumin-yeast extract medium. The specimens were sent to the laboratory by regular mail service. Upon receipt, routine viral diagnostics were carried out, and 1.0 ml was stored at -70°C for testing in the *Mycoplasma* group-specific PCR assay and the *M. pneumoniae*-specific PCR assay (17).

Culture of chlamydial strains. HeLa 229 cells (ATCC CCL 2.1) were used for propagation of *C. trachomatis* strains, as previously described (9). Iscove's mod-

* Corresponding author. Mailing address: Research Laboratory for Infectious Diseases, RIVM, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Phone: 31 30 2743942. Fax: 31 30 2744449. Electronic mail address: JM.Ossewaarde@rivm.nl.

TABLE 1. Location of primers and the probe used in the *Mycoplasma* group-specific PCR assay and corresponding sequences of *M. arginini* and three chlamydial species in the complete 16S rRNA gene

Origin	Location of primers and probe in the 16S rRNA gene ^a		
	Upstream primer GPO-3 (positions 774–798)	Probe GPO-4 (positions 910–933)	Downstream primer MGSO (positions 1029–1055) ^b
Oligonucleotide	5'-GGGAGCAAACAGGATTAGATACCCT-3'	5'-CTTAAAGGAATTGACGGGAACCCG-3'	5'-GAGGTTAACAGAGTGACAGATGGTGCA-3'
<i>M. arginini</i>G.....G.....G.....
<i>C. pneumoniae</i>C...A.....GG....	CGCAAGG.....TAC....G...C....
<i>C. trachomatis</i>C...A.....GG....	CGCAAGG.....TAC....G...C....
<i>C. psittaci</i>C...A.....GG....	CGCAAGG.....TAC....G...C....

^a Location numbering from reference 19. A dot represents a nucleotide identical to that in the oligonucleotide sequence.

^b Note that the actual primer sequence is the complementary sequence.

ified Dulbecco medium (GIBCO) supplemented with 10% fetal calf serum and antibiotics was used. One-day-old monolayers in shell vials and 24-well microtiter plates were used. All monolayers were pretreated with DEAE-dextran. The vials and microtiter plates were centrifuged for 1 h at $4,800 \times g$ at 25°C and incubated at 37°C in 5% CO₂ for 3 days. The microtiter plates were fixed with methanol and stained with fluorescein-labeled anti-lipopolysaccharide (LPS) monoclonal antibodies (PathoDx; Diagnostic Products Corp., Los Angeles, Calif.) to assess the presence of inclusions. Suspensions were prepared from the monolayers in the shell vials after the medium was replaced with fresh cold medium by ultrasonic treatment for 1 min in a cup horn (Vibra Cell; Sonics & Materials, Inc., Danbury, Conn.). *C. pneumoniae* and *C. pecorum* strains were propagated by the same method but without pretreatment of the monolayers with DEAE-dextran. HEP-2 cells were used for propagation of *C. pneumoniae* (15).

Decontamination procedures. Three different decontamination procedures were used. In the first procedure, 10% heifer-specific antiserum against *M. arginini* was used in place of fetal calf serum (11) and *Chlamydia* strains were subcultured in four passages. This antiserum was raised against *M. arginini* 10129, was not inactivated, and was stored at -70°C (11). It reacted positively in a growth inhibition test on agar (11) and in an immunofluorescence test with agar colonies. All subpassages were tested for the presence of *Mycoplasma* DNA by PCR. In the second procedure, chlamydial suspensions were centrifuged for 20 min at $14,000 \times g$ in a microcentrifuge, resuspended in trypsin solution, and incubated for 30 min at 37°C. Next, the suspensions were centrifuged through a layer of 35% sodium diatrizoate, resuspended in 10 mM phosphate-buffered saline, pH 7.2 (PBS), with 30 µg of DNase I per ml for cell culture (Boehringer-Mannheim, Mannheim, Germany), and incubated for 60 min at 37°C. After centrifugation through a layer of 35% sodium diatrizoate, the pellets were resuspended in medium and used to inoculate monolayers. After treatment and subpassage, the suspensions were tested for the presence of *Mycoplasma* DNA by PCR. In the third procedure, chlamydial suspensions were centrifuged, and the pellets were resuspended in PBS with 1% Triton X-100 or 1% Tween 20 and incubated at 4°C for 30 min. Next, the suspensions were centrifuged, resuspended in 25 µl of medium, and transferred to a clean tube with SM2 beads (Bio-Rad, Veenendaal, The Netherlands) equivalent to approximately 50 µl to remove traces of Triton X-100, which is toxic to cell cultures. Medium was added to a volume of 500 µl, and the tubes were incubated at 4°C for 30 min. After centrifugation, the pellets were resuspended in medium and used to inoculate monolayers. After treatment and subpassage, the suspensions were tested for the presence of *Mycoplasma* DNA by PCR.

***Mycoplasma*-specific PCR.** Prevention of molecular contamination was as previously described (9). The nucleotide sequences of primers and probe used in the *Mycoplasma* group-specific PCR assay are as follows: upstream primer GPO-3, 5'-GGGAGCAAACAGGATTAGATACCCT-3'; downstream primer MGSO, 5'-TGCACCATCTGTCACTCTGTTAACTC-3'; and probe GPO-4, 5'-CTTAAAGGAATTGACGGGAACCCG-3'. Table 1 shows the locations of the primers and the probe and their corresponding chlamydial nucleotide sequences relative to those of the complete 16S rRNA gene. The PCR method used was basically as described by Van Kuppeveld et al. (19). In short, a suspension of 250,000 to 500,000 HEP-2 cells, 0.5 to 1.0 ml of sonicated chlamydial culture, or 1.0 ml of throat swab transport medium was centrifuged for 20 min at $14,000 \times g$ in a microcentrifuge. The supernatant was discarded, and the pellet was lysed in a buffer with proteinase K (9). Five microliters of sample was added to 45 µl of the following PCR mixture (final concentrations): 50 mM Tris-HCl (pH 8.5 at 37°C), 50 mM NaCl, 2 mM MgCl₂, 10 µM tetramethylammonium chloride, 0.01% bovine serum albumin, 1.5 U of AmpliTaq (Perkin-Elmer, Gouda, The Netherlands), 200 µM (each) deoxynucleoside triphosphate, 1 µM upstream primer GPO-3, and 1 µM downstream primer MGSO (19). The mixture was overlaid with 2 drops of mineral oil. A touchdown PCR protocol (3) was used in an Omnigene thermal cycler (Hybaid; Biozym, Landgraaf, The Netherlands), with denaturation at 94°C for 1 min, extension at 72°C for 1 min, and initially, two cycles of annealing at 65°C for 1 min each. Every two cycles, the annealing temperature was lowered by 2° until it reached 55°C. At this annealing temperature, 30 cycles were carried out. Ten microliters of each sample was analyzed by

agarose electrophoresis, using marker VIII as a reference (Boehringer-Mannheim), and photographed under UV light after being stained with ethidium bromide. Initially, the specificity of the results was confirmed by blotting the gels onto nylon membranes, hybridizing with the biotinylated oligonucleotide GPO-4 (19), and after reaction with peroxidase-labeled streptavidin (Boehringer-Mannheim), visualizing with the ECL kit (Amersham, 's-Hertogenbosch, The Netherlands). Later, only direct photography of ethidium bromide-stained gels was used. Throat swabs from patients were also tested by an *M. pneumoniae*-specific PCR assay for the presence of *M. pneumoniae*-specific DNA (17).

Automated sequencing. The PCR products of two different contaminated chlamydia strains were sequenced in an automated sequencer (ABI, Gouda, The Netherlands) with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (ABI). The derived sequence was compared with known sequences in the GenBank database by using the BLAST server developed by the National Center for Biotechnology Information at the National Library of Medicine (1).

Culture and identification of *Mycoplasma* spp. Samples of *Chlamydia* strains and cell lines were frozen immediately after sampling and thawed directly before inoculation for enrichment in broth and growth on agar. Modified Herderschée and Difco PPLO media were both used as culture media, as described previously (12). Four tubes each containing 5 ml of each medium were inoculated with 0.5 ml of sample, and four plates were inoculated with 0.1 ml each. Half of the used media was incubated aerobically and the other half was incubated in an atmosphere of 95% N₂-5% CO₂. The broth cultures were subcultured on agar after 7 and 14 days and further incubated in the respective atmosphere. All cultures were incubated at 37°C. Agar plates were examined weekly for 3 weeks. Colonies appearing on agar plates were identified by indirect immunofluorescence, using specific polyclonal rabbit antibodies and horse anti-rabbit immunoglobulins labelled with fluorescein isothiocyanate (16). In parallel with this culture procedure, DNA staining was carried out by using indicator cells, as described previously (12).

RESULTS

Detection and confirmation of *Mycoplasma* contamination. The cell lines were tested by culture for contamination by *Mycoplasma* spp. Samples from three different cell lines were positive by DNA staining. A *Mycoplasma* sp. was cultured from these samples and serologically typed as *M. arginini* by indirect immunofluorescence. Samples from these cell lines were also tested by the *Mycoplasma* group-specific PCR assay. A product of approximately 270 bp was visible on agarose gels. Hybridization with the biotinylated oligonucleotide GPO-4 was positive. Next, suspensions from cultures of *C. pneumoniae* CWL-011 and CWL-050 were tested by the *Mycoplasma* group-specific PCR assay. A product of the expected size was visible on agarose gel, and a positive signal was obtained after hybridization with the biotinylated oligonucleotide GPO-4. To confirm the specificity of the *Mycoplasma* group-specific PCR assay, the DNA nucleotide sequence of the amplified product was determined by automated sequencing. Amplified products from two contaminated strains, CWL-011 and CM-1, were sequenced, each in both directions. The four nucleotide sequences obtained were identical. The BLAST server retrieved the 16S rRNA sequence of *M. arginini* and that of *M. gateae*, both with 100% identity to our PCR product. Figure 1 shows

<i>M. arginini</i>	GGGAGCAAC AGGATTAGAT ACCCTGGTAG TCCAGCCCGT AAACGATGAT
PCR productTT.....CA
<i>C. pneumoniae</i>TT.....CA
<i>C. trachomatis</i>TT.....CA
<i>C. psittaci</i>TT.....CA
<i>M. arginini</i>	CATTAGTCGG TGGAGACTTC AC-----TGACGCAGC TAACGCATTA
PCR productTG.....G...
<i>C. pneumoniae</i>	T.C.T.A.T.T G.AT.GTC...ACCCCATCC G..T..G...TG...
<i>C. trachomatis</i>	T.C.T.A.T.T G.AT.GTC...ACCCCATCC G..T..G...G...
<i>C. psittaci</i>	T.C.T.A.T.T G.ATAGTC...ACCCATCC G..T..T...G...
<i>M. arginini</i>	AATGATCCGC CTGACTAGTA TGCTCGCAAG ACTGAAACTT AAGGAATTG
PCR productG.....CA.....G.....C.....A.....
<i>C. pneumoniae</i>G.....CA.....G.....C.....A.....
<i>C. trachomatis</i>G.....CA.....G.....C.....A.....
<i>C. psittaci</i>G.....CA.....G.....C.....A.....
<i>M. arginini</i>	ACGGGGACCC GCACAAGCGG TGGAGCATGT GGTITATTT GAAGATACGC
PCR productG.....A.....C.....T.CA.....
<i>C. pneumoniae</i>G.....A.....C.....T.CA.....
<i>C. trachomatis</i>G.....A.....C.....T.CA.....
<i>C. psittaci</i>G.....A.....C.....T.CA.....
<i>M. arginini</i>	GGAGAACCCTT ACCCACTCTT CACATCCTTC GCAATGCTAT AGAGATATAG
PCR productGTA.T TG.CAA..G...A..C..
<i>C. pneumoniae</i>GTA.T TG.CCA..GGC...A..G.C..
<i>C. trachomatis</i>GTA.T TG.CCA..GGC...A..G.C..
<i>C. psittaci</i>GTA.T TG.CCA..GGC...A..G.C..
<i>M. arginini</i>	CA--GAGGT TAACCGAGTC ACAGATGGTG CA
PCR productTTTCGCAA GG..A..TACG..C..
<i>C. pneumoniae</i>TTTCGCAA GG..A..TACG..C..
<i>C. trachomatis</i>TTTCGCAA GG..A..TACG..C..
<i>C. psittaci</i>TTTCGCAA GG..A..TACG..C..

FIG. 1. Aligned sequences of *M. arginini* (GenBank accession number U15794), the PCR-amplified product, *C. pneumoniae* (GenBank accession number L06108), *C. trachomatis* (GenBank accession number M59178), and *C. psittaci* (GenBank accession number M13769). A dot represents a nucleotide identical to that in the first-mentioned sequence. A dash represents a gap introduced for alignment. The locations of the primers and probe are underlined in the *M. arginini* sequence.

the derived sequence aligned with the known sequences from *M. arginini*, *C. pneumoniae*, *C. trachomatis*, and *C. psittaci*.

Results of decontamination procedures. All procedures were first tested with *C. trachomatis* L2. The strain was first propagated in contaminated cells. Next, the strain was passaged in medium containing 10% *M. arginini*-specific anti-serum. This procedure did not affect the presence of *Mycoplasma* spp. Also, treatment with trypsin and DNase I was not effective. Next, contaminated suspensions of the L2 strain were treated with 1% Triton X-100 or 1% Tween 20. Directly after treatment, there was visibly less PCR product on agarose gel electrophoresis than with the untreated control. After two series of treatments, the suspension treated with Tween 20 was still *Mycoplasma* positive by the PCR assay, but the suspension treated with Triton X-100 was *Mycoplasma* negative. *Mycoplasma* cultures of this suspension were also negative. Next, Triton X-100 treatment was tested on a number of contaminated strains: *C. trachomatis* serovar D, *C. pneumoniae* TW-183, CM-1, 2023, 2043, CWL-011, CWL-029, CWL-050, GRO-21, IOL-207, and UZG-1, and *C. pecorum* E58. Usually all strains were *Mycoplasma* DNA negative after one treatment and subpassage. One strain, CWL-011, was *Mycoplasma* DNA positive after the first treatment but negative after the second treatment. However, after two passages in noncontaminated cells, the strain was *Mycoplasma* DNA positive again. Two additional treatments with Triton X-100 permanently rid the strain of *Mycoplasma* contamination. Figure 2 shows an agarose gel of the PCR products of strains CWL-011, CM-1, and GRO-21 before treatment, after the first treatment with Triton X-100, and after the second treatment and subpassage. All but

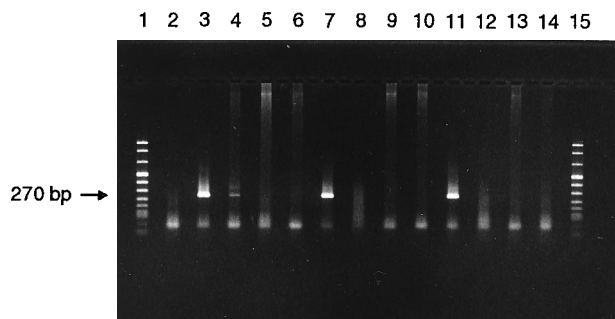


FIG. 2. Agarose gel of PCR products from three contaminated *C. pneumoniae* strains. The lanes were loaded as follows: 1 and 15, marker VIII; 2, 6, 10, and 14, negative control; 3, 4, and 5, CWL-011 before, after the first, and after the second treatment; 7, 8, and 9, CM-1 before, after the first, and after the second treatment; 11, 12, and 13, GRO-21 before, after the first, and after the second treatment. The arrow indicates the position of the amplified 270-bp fragment.

three strains showed >75% of the inclusions of untreated suspensions after Triton X-100 treatment. Two strains, strains 2023 and 2043, showed a 100-fold reduction in number of inclusions and one strain, UZG-1, was not viable after Triton X-100 treatment and could not be decontaminated in this way.

Mycoplasma DNA detection in clinical specimens. Throat swabs from 40 patients with symptoms of an upper respiratory tract infection were tested for the presence of *Mycoplasma* DNA. Thirty-nine specimens reacted positively in the *Mycoplasma* group-specific PCR assay. Of these 39 specimens, 1 reacted positively in the *M. pneumoniae*-specific PCR assay; all other specimens reacted negatively. No attempts to type these *Mycoplasma* strains further were made.

DISCUSSION

Contamination of biological materials with *Mycoplasma* spp. may alter several parameters of many sorts of experiments (7). Therefore, when these parameters are studied, it is essential to use *Mycoplasma*-free materials. For example, when random DNA amplification techniques with nonspecific primers are used, the template DNA should be of the highest purity possible. Although we could eliminate *Mycoplasma* DNA, as determined by PCR, from suspensions of chlamydial elementary bodies by differential centrifugation, enzymatic treatment with trypsin and DNase, and repeated centrifugation on a layer of 35% sodium diatrizoate (data not shown), it is advisable to start with *Mycoplasma*-free material. Most contaminations result from the use of infected cell cultures, although *Mycoplasma* spp. may be coisolated from clinical specimens taken from sites harboring *Mycoplasma* spp. In our laboratory we were faced with *Mycoplasma*-contaminated cell lines in July 1994. Although these cell lines were easily replaced by noncontaminated cell lines stored in liquid nitrogen and most chlamydial strains could be propagated from their original vials in a *Mycoplasma*-free environment, some strains could not be rescued. Recently, Messmer et al. reported *Mycoplasma* contamination in several *C. pneumoniae* strains obtained from different laboratories (8). Strains were found contaminated with *M. hyorhinitis*, *M. hominis*, and one unidentified species. These authors argued that contamination might have occurred as a result of the use of contaminated cell lines or because of the presence of *Mycoplasma* spp. in the original specimen. Therefore, we decided to develop a method for removing *Mycoplasma* contamination from suspensions of *Chlamydia* spp.

M. arginini was cultured from our contaminated cell lines.

Since many of the primer sets for detection of *Mycoplasma* spp. described in the literature cross-react with *Chlamydia* spp., we confirmed the PCR assay results by sequencing the PCR product. This sequence was identical to the *M. arginini* sequence retrieved from GenBank. However, sequences should be classified carefully. Initially, *M. arginini* 16S rRNA sequences were not available in the GenBank database, and the BLAST server retrieved the 16S rRNA sequence of *M. arthritidis* as the sequence with the highest percentage of identity with our PCR product. At the time our experiments were carried out, the only available *M. arginini* sequence contained 58 unknown nucleotides within our amplified fragment. At the time of preparation of this paper, the BLAST server retrieved the 16S rRNA sequence of *M. arginini* and that of *M. gateae*, both with 100% identity to our PCR product.

No product was seen on gel when noncontaminated cell lines or *Chlamydia* suspensions were used. Apparently, the specificity of the downstream primer was sufficient. Next, we used this PCR assay to monitor several decontamination procedures.

Three methods for decontamination were examined. Treatment of *Mycoplasma*-contaminated cell cultures with antibiotics eliminates this microorganism in many cases, but in some cases resistant *Mycoplasma* strains emerge and even death of cell lines may occur (4). Since antibiotics active against *Mycoplasma* spp. are also active against chlamydiae, they could not be used. Because most chlamydial strains need centrifugation-assisted infection, removal of *Mycoplasma* spp. must be very thorough; otherwise, *Mycoplasma* particles may be forced inside cells and survive treatment. With a specific antiserum, *Chlamydia* strains could not be cleared of *Mycoplasma* spp. Complement activity in this serum was not determined. Previous studies showed this antiserum to be effective in curing cell lines from contamination with *M. arginini* only in combination with antibiotics (11). Enzymatic treatment also was not successful, although a more laborious procedure was successful in removing *Mycoplasma* DNA from suspensions of elementary bodies (data not shown). Next, we exploited the fact that mycoplasmas have a cell membrane consisting only of a lipid bilayer. This membrane is not resistant to the organic solvents ether and chloroform (6). Since bacteria are usually sensitive to ether or chloroform treatment, we used detergents to dissolve the membranes. Tween 20 proved not to be strong enough, but Triton X-100 was successful in removing the *Mycoplasma* contamination from all strains tested except one. This strain, UZG-1, isolated from a patient in Belgium, belonged to the species *C. pneumoniae*, since it reacted with antichlamydial LPS monoclonal antibodies, with the *C. pneumoniae*-specific monoclonal antibody RR402, and with a *C. pneumoniae*-specific 16S rRNA PCR. Even after a short incubation with 0.05 to 1.0% Triton X-100, no viable elementary bodies remained in the suspension. The reason for this physical difference among *C. pneumoniae* isolates needs to be studied further. Whether or not treatment with Triton X-100 selects a resistant subpopulation with possibly different characteristics was not tested further.

It is very difficult to propagate clinical isolates of *C. pneumoniae*. Most strains are lost after only a few passages. We observed a large difference in growth characteristics among *C. pneumoniae* isolates in *Mycoplasma*-free and *Mycoplasma*-contaminated cell lines. In the latter, less cells were infected, the number of inclusions was smaller, and the size of the inclusions was significantly smaller. Considering that 39 of 40 throat swab specimens reacted positively in the *Mycoplasma* group-specific

PCR assay, all but one contaminated with *Mycoplasma* species other than *M. pneumoniae*, it is very possible that contamination of clinical specimens with *Mycoplasma* spp. prohibits the successful isolation and propagation of *C. pneumoniae*. In this case, monolayers of the first and second passages of clinical specimens in cell lines could be treated blindly with Triton X-100 to remove the *Mycoplasma* contamination, allowing successful isolation of more *C. pneumoniae* strains.

In conclusion, we have developed a method that uses Triton X-100 to rid *Chlamydia* strains of *Mycoplasma* contamination, as confirmed by culture and a *Mycoplasma* group-specific PCR assay.

ACKNOWLEDGMENT

We thank J. C. de Jong for critical comments during the preparation of the manuscript.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Carroll, K., and R. O'Kennedy. 1988. The elimination of *Mycoplasma* from infected hybridomas by passaging in BALB/c mice. *J. Immunol. Methods* **108**:189–193.
- Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**:4008.
- Drexler, H. G., S. M. Gignac, Z.-B. Hu, A. Hoppert, E. Fleckenstein, M. Voges, and C. C. Uphoff. 1994. Treatment of *Mycoplasma* contamination in a large panel of cell cultures. *In Vitro Cell Dev. Biol.* **30A**:344–347.
- Eremeeva, M. E., N. M. Balayeva, and D. Raoult. 1994. Purification of Rickettsial cultures contaminated by *Mycoplasmas*. *Acta Virol.* **38**:231–233.
- La Linn, M., A. J. D. Bellett, P. G. Parsons, and A. Suhrbier. 1995. Complete removal of *Mycoplasma* from viral preparations using solvent extraction. *J. Virol. Methods* **52**:51–54.
- McGarrity, G. J., V. Vanaman, and J. Sarama. 1984. Cytogenic effects of mycoplasma infection of cell cultures: a review. *In Vitro* **20**:1–18.
- Messmer, T. O., C. M. Black, and W. L. Thacker. 1994. *Mycoplasma* contamination of *Chlamydiae* isolated from clinical specimens. *APMIS* **102**:793–796.
- Ossewaarde, J. M., M. Rieffe, M. Rozenberg-Arska, P. M. Ossenkoppele, R. P. Nawrocki, and A. M. van Loon. 1992. Development and clinical evaluation of a polymerase chain reaction test for detection of *Chlamydia trachomatis*. *J. Clin. Microbiol.* **30**:2122–2128.
- Polak-Vogelzang, A. A., A. F. Angulo, J. Brugman, and R. Reygers. 1990. Survival of *Mycoplasma hyorhinis* in trypsin solutions. *Biologicals* **18**:97–101.
- Polak-Vogelzang, A. A., J. Brugman, A. D. M. E. Osterhaus, and R. Reygers. 1987. Elimination of *Mycoplasma* from cell cultures by means of specific bovine antiserum. *Zentralbl. Bakteriell. Hyg. Reihe A* **264**:84–92.
- Polak-Vogelzang, A. A., J. Brugman, and R. Reijgers. 1987. Comparison of two methods for detection of *Mollicutes* (*Mycoplasma* and *Acholeplasma*) in cell cultures in The Netherlands. *Antonie van Leeuwenhoek* **53**:107–118.
- Ravaoariroro, M., and J. Lecomte. 1988. Evaluation of three methods for curing hybridomas from *Mycoplasma* contamination. *Hybridoma* **7**:79–86.
- Rawadi, G., and O. Dussurget. 1995. Advances in PCR-based detection of mycoplasmas contaminating cell cultures. *PCR Methods Appl.* **4**:199–208.
- Roblin, P. M., W. Dumornay, and M. R. Hammerschlag. 1992. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* **30**:1968–1971.
- Rosendal, S., and F. T. Black. 1972. Direct and indirect immunofluorescence of unfixed and fixed *Mycoplasma* colonies. *Acta Pathol. Microbiol. Scand. Sect. B Microbiol.* **80**:615–622.
- Ursi, J.-P., D. Ursi, M. Ieven, and S. R. Pattyn. 1992. Utility of an internal control for the polymerase chain reaction. Application to detection of *Mycoplasma pneumoniae* in clinical specimens. *APMIS* **100**:635–639.
- Van Kuppeveld, F. J. M., K.-E. Johansson, J. M. D. Galama, J. Kissing, G. Bölske, J. T. M. van der Logt, and W. J. G. Melchers. 1994. Detection of *Mycoplasma* contamination in cell cultures by a *Mycoplasma* group-specific PCR. *Appl. Environ. Microbiol.* **60**:149–152.
- Van Kuppeveld, F. J. M., J. T. M. van der Logt, A. F. Angulo, M. J. van Zoest, W. G. V. Quint, H. G. M. Niesters, J. M. D. Galama, and W. J. G. Melchers. 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl. Environ. Microbiol.* **58**:2606–2615. (Erratum, **59**:655, 1993.)